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Authors: Hiroi, Junya, Kaneko, Toyoji, Seikai, Tadahisa, and Tanaka, Masaru

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Developmental Sequence of Chloride Cells in the Body Skin and Gills of Japanese Flounder (*Paralichthys olivaceus*) Larvae

Junya Hiroi[1,2], Toyoji Kaneko[2], Tadahisa Seikai[3] and Masaru Tanaka[1]*

[1]*Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo, Kyoto 606-8502, Japan*
[2]*Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164-8639, Japan*
[3]*Fisheries Research Station, Kyoto University, Maizuru, Kyoto 625-0086, Japan*

ABSTRACT—The developmental sequence of chloride cells was examined in both the body skin and gills of Japanese flounder (*Paralichthys olivaceus*) larvae by whole-mount immunocytochemistry using an antiserum specific for Na^+,K^+-ATPase. In premetamorphic larvae at 0 and 4 days after hatching (days 0 and 4), immunoreactive chloride cells were distributed only in the yolk-sac membrane and body skin. Premetamorphic larvae at days 8-18 possessed both cutaneous and branchial chloride cells. Large chloride cells in the skin of premetamorphic larvae often formed multicellular complexes, suggestive of their ion-secreting function. Cutaneous chloride cells decreased in size and density at the beginning of metamorphosis (days 21 and 24), and disappeared at the metamorphic climax (days 28 and 33). In contrast, branchial chloride cells first appeared at day 8, and increased during metamorphosis. These results indicate that the site for ion secretion in seawater may shift from cutaneous to branchial chloride cells during metamorphosis. The appearance of branchial chloride cells before the differentiation of gill lamellae suggests that the primary function of the gills during the early development is ion regulation rather than gas exchanges.

INTRODUCTION

Embryos and larvae of teleosts maintain the internal hydromineral balance, although important osmoregulatory organs in adults such as the gills, kidney and intestine are not fully developed (Alderdice, 1988). In juveniles and adults of marine teleosts, chloride cells in the gills are responsible for the secretion of excess Na^+ and Cl^- in the body fluids (Zadunaisky, 1984). In embryos and larvae, chloride cells located in the epithelia covering the yolk and body have been suggested as ion-secreting sites in seawater (Shelbourne, 1957; Lasker and Threadgold, 1968; Hwang and Hirano, 1985; Alderdice, 1988; Hwang, 1989, 1990; Ayson *et al*., 1994; Kaneko *et al*., 1995; Shiraishi *et al*., 1997; Sasai *et al*., 1998a). Thus, these extrabranchial chloride cells as the major site for ion secretion seem to be taken over by branchial chloride cells as fish grow. However, the spatial shift of chloride cell distribution from the yolk-sac membrane and body skin to gills and their developmental sequence have not been demonstrated through the early development of teleosts.

Marine teleosts generally undergo physiological changes as well as morphological and ecological changes during metamorphosis from larvae to juveniles. Japanese flounder (*Paralichthys olivaceus*) exhibit drastic metamorphosis from pelagic larvae to benthic juveniles, involving the migration of the right eye to the left side of the head. In the present study, to clarify the spatial shift of chloride cell distribution during metamorphosis, we examined the development of both cutaneous and branchial chloride cells in premetamorphic and metamorphic flounder larvae. To detect chloride cells, whole larvae were subjected to immunocytochemical staining with an antiserum specific for Na^+,K^+-ATPase, a key enzyme of ion transport in chloride cells.

MATERIALS AND METHODS

Fish

Naturally spawned eggs of Japanese flounder (*Paralichthys olivaceus*) were collected from a brood-stock tank in the Fisheries Research Station of Kyoto University. Larvae were reared in a polycarbonate tank (500 l) with running seawater. Water temperature was maintained at 18°C, and the salinity ranged between 30.6 and 32.2 ppt. They were initially fed on rotifers (*Brachionus plicatilis*) cultivated with *Nannochrolopsis* sp. and ω-Yeast (Kyowa Hakko Kogyo, Japan), and later on brine shrimp (*Artemia* spp.) nauplii enriched with Ester-85 (Nippon Chemical Feed, Japan). Samples were taken at nine different developmental stages (Table 1). The larvae up to 18 days after

* Corresponding author: Tel. +81-75-753-6220; FAX. +81-75-753-6229.

Table 1. Relative frequency of chloride cells in the body skin and gills during the early development of Japanese flounder

Days after hatching	Developmental stage[1]	Body length (mm)	Chloride cell frequency[2]	
			skin	gills
premetamorphic larvae				
0		2.5 - 2.6	+	–
4	A	2.8 - 3.1	++	–
8	B	4.0 - 4.3	++	+
14	C	5.0 - 5.5	++	++
18	D	5.8 - 6.1	++	++
metamorphic larvae				
21	E	6.8 - 7.2	+	++
24	F	7.3 - 7.5	±	++
28	G	8.0 - 8.9	–	++
33	H	9.5 - 10.2	–	++

[1] Developmental stages according to Minami (1982).
[2] –, not detected; ±, sparse; +, moderately dense; ++, dense.

hatching (day 18) with symmetrical bodies were regarded as premetamorphic larvae, and those on and after day 21 with migrating right eyes as metamorphic larvae. Larvae were anesthetized with MS-222, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 hr at 4°C, and preserved in 70% ethanol.

Whole-mount immunocytochemistry

As a specific probe for chloride cells, we used an antiserum specific for Na^+,K^+-ATPase, which is localized in the membrane of the tubular systems distributed extensively over the cytoplasm of chloride cells (Karnaky *et al.*, 1976; Hootman and Philpott, 1979). The antiserum was raised in a rabbit against a synthetic peptide corresponding to part of the highly conserved region of the Na^+,K^+-ATPase α-subunit (Ura *et al.*, 1996). It has been well documented that the anti-Na^+,K^+-ATPase specifically detects both cutaneous and branchial chloride cells in several teleosts (Ura *et al.*, 1996; Uchida *et al.*, 1996; Shiraishi *et al.*, 1997; Sasai *et al.*, 1998a, b).

Whole-mount immunocytochemistry based on the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.*, 1981) was carried out following the method of Ohtani *et al.* (1989) using commercial reagents (Vectastain Elite ABC kit, Vector Laboratories, USA). The right operculum of larvae after day 4 was removed prior to the immunostaining in order to enhance penetration of solutions to the gills. After treatment with 0.1% sodium cyanoborohydride in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 1 hr, the samples were incubated sequentially with: 1) rabbit anti-Na^+,K^+-ATPase diluted 1:500 for 20 hr at 4°C, 2) biotinylated goat anti-rabbit IgG for 20 hr at 4°C, 3) ABC reagent for 20 hr at 4°C, and 4) 0.03% 4-Cl-1-naphthol in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.003% hydrogen peroxide for 20 min. The antisera and ABC reagent were diluted with PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin and 0.01% sodium azide. The whole sample and separated gill arches were mounted on a slide with glycerin.

The image of the body surface on the left side was digitized with a CCD video camera (Victor, Japan) and an image processor (ARGUS 20, Hamamatsu photonics, Japan), and the size and density of immunopositive chloride cells in the body skin were measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/). The quantitative analysis was made on cutaneous chloride cells only in the yolk-sac membrane and the skin of trunk and tail, in which chloride cells were uniformly distributed during early developmental stages. The head region, finfold of premetamorphic larvae and fins of metamorphic larvae were excluded from the measurement, because the sparseness of chloride cells in these regions was considered inappropriate for density measurement. The quantitative analysis of branchial chloride cells was not carry out, since it was difficult to measure the precise cell density and size in the complicated, three-dimensional structures of the gills.

RESULTS

Chloride cells in the skin

In newly-hatched flounder larvae (day 0), weakly-immunopositive but large chloride cells were detected in the epithelia covering both the yolk and body (Fig. 1A, B). In premetamorphic larvae at days 4-18, a large number of immunopositive chloride cells were distributed in the body skin (Fig. 1C-F). Chloride cells in the skin of newly-hatched and premetamorphic larvae were characterized by the formation of cell clusters: several chloride cells congregated to form a multicellular complex as indicated by the presence of more than one immunonegative nuclei (Fig. 1B, D, F). Especially, large chloride cell complexes were observed in the abdominal region. In metamorphic larvae at day 21 just starting the right-eye migration, both size and density of chloride cells decreased (Fig. 1G, H). In contrast with premetamorphic larvae, these chloride cells were present individually and did not form multicellular complexes. No chloride cells were detected in the body skin of metamorphic-climax larvae at days 28 and 33. However, small immunopositive cells not forming cellular complexes were found in the pectoral, dorsal and anal fins of metamorphic larvae at days 21-33, although few chloride cells were detectable in the pectoral fin and finfold of premetamorphic larvae at days 0-18. Figure 2 shows developmental changes in size-frequency distributions of chloride cells and chloride cell complexes in the yolk-sac membrane and body skin. During the early development, the mean size of chloride cells decreased concomitant with the disappearance of chloride cell complexes. The density of chloride cells was decreased drastically at the beginning of metamorphosis (days 21 and 24), and the cells disappeared at the metamorphic climax at days 28 and 33 (data not shown).

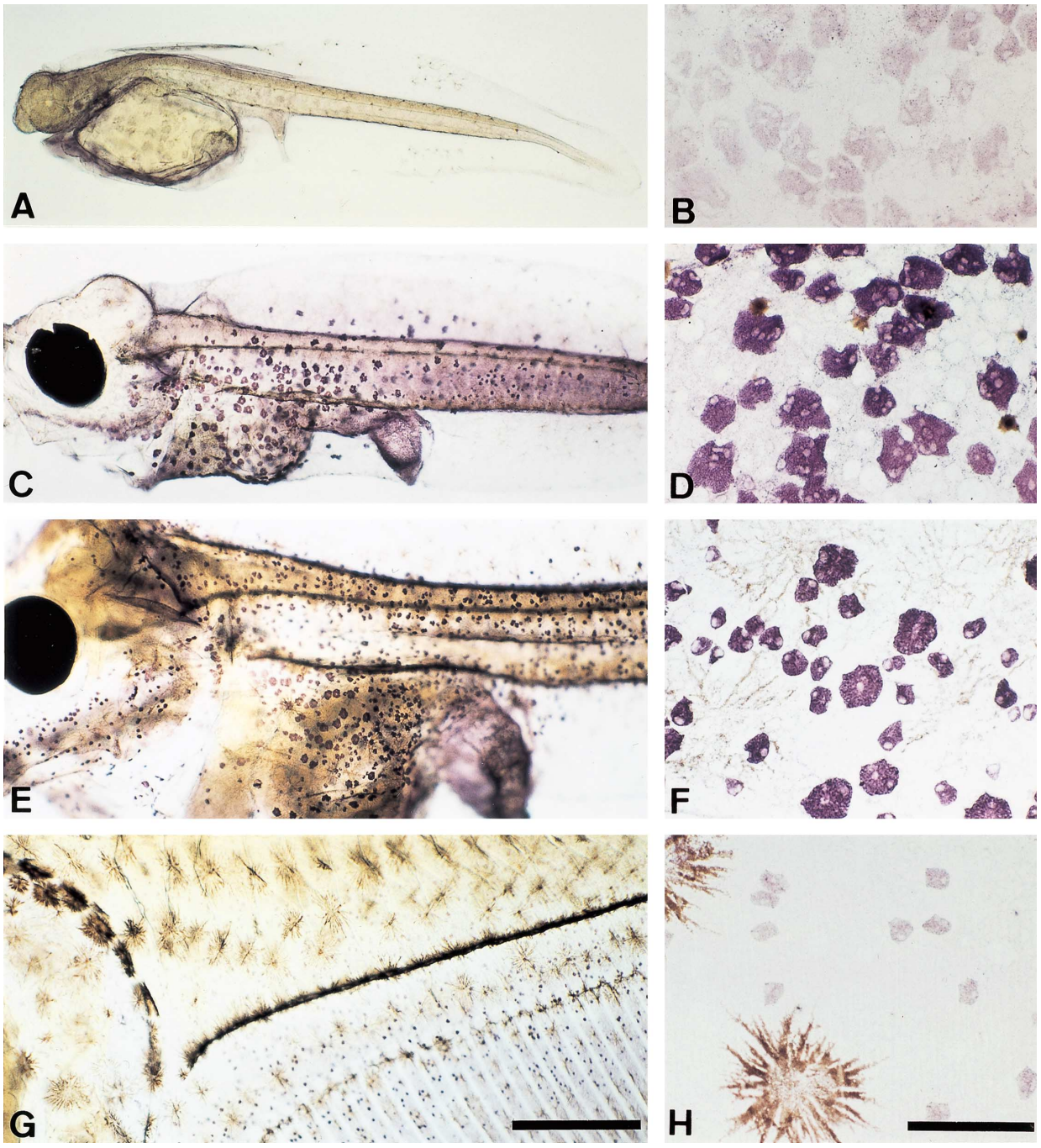

Fig. 1. Cutaneous chloride cells of flounder larvae at day 0 (**A**, **B**), day 8 (**C**, **D**), day 18 (**E**, **F**) and day 24 (**G**, **H**), detected by whole-mount immunocytochemistry using an antiserum specific for Na^+,K^+-ATPase. (**B**, **D**, **F**, **H**) Magnified views of separated yolk-sac membrane or body skin in the abdominal region. Bars: (**A**, **C**, **E**, **G**) 500 μm; (**B**, **D**, **F**, **H**) 100 μm.

Chloride cells in the gills

The gills were not distinguishable in the whole-mount preparation of newly-hatched larvae at day 0. Four pairs of gill arches were recognizable in larvae at day 4, although the gill filament and lamella were not yet differentiated. Chloride cells were not yet detected in the gill arches at this early developmental stage. In premetamorphic larvae at day 8, gill filaments sprouted from the gill arches but the lamella was not differentiated. Immunoreactive chloride cells were observed in the filament epithelia of all the four pairs of gills (Fig. 3A). In premetamorphic larvae at day 18, the filaments were further developed and the lamellae were extended from the filaments. Chloride cells were extensively distributed in the filaments, but were absent in the lamellae (Fig. 3B). In metamorphic larvae at day 24, when the filaments and lamellae were extended further, chloride cells were overspread in the filaments, whereas no chloride cell was observed in the lamellae (Fig. 3C).

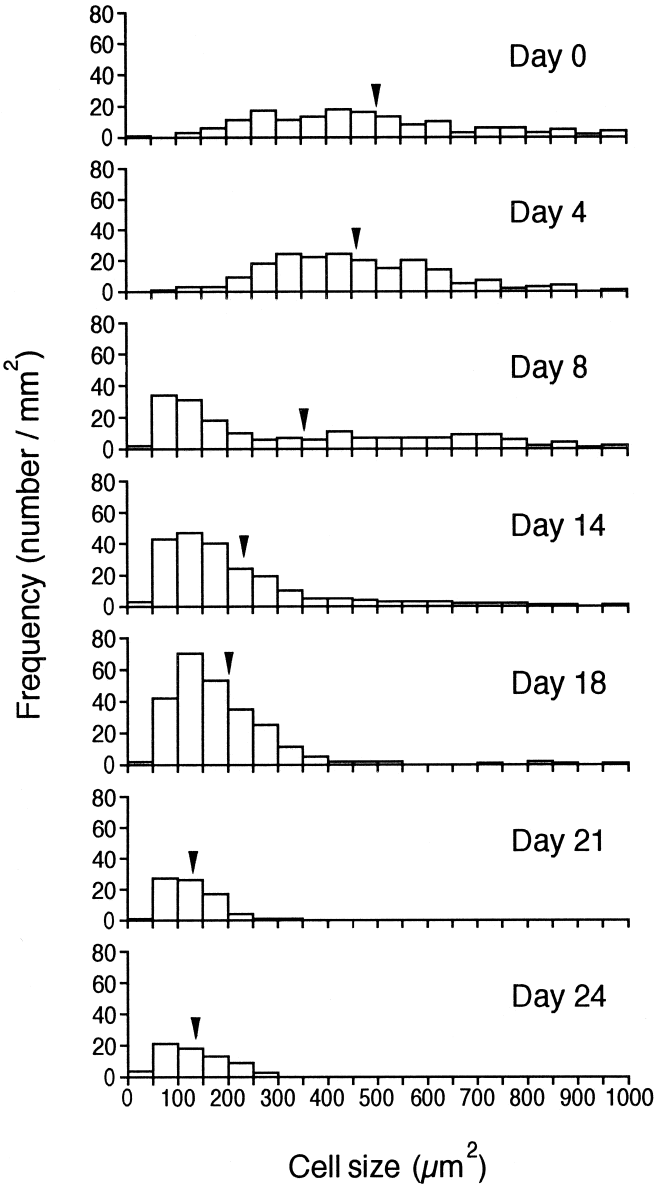

Fig. 2. Developmental changes in size-frequency distributions of chloride cells in the yolk-sac membrane and body skin of flounder larvae. Data obtained from 4 individuals were combined at each day. Arrows indicate the mean values. No chloride cell is detectable in the body skin at days 28 and 33.

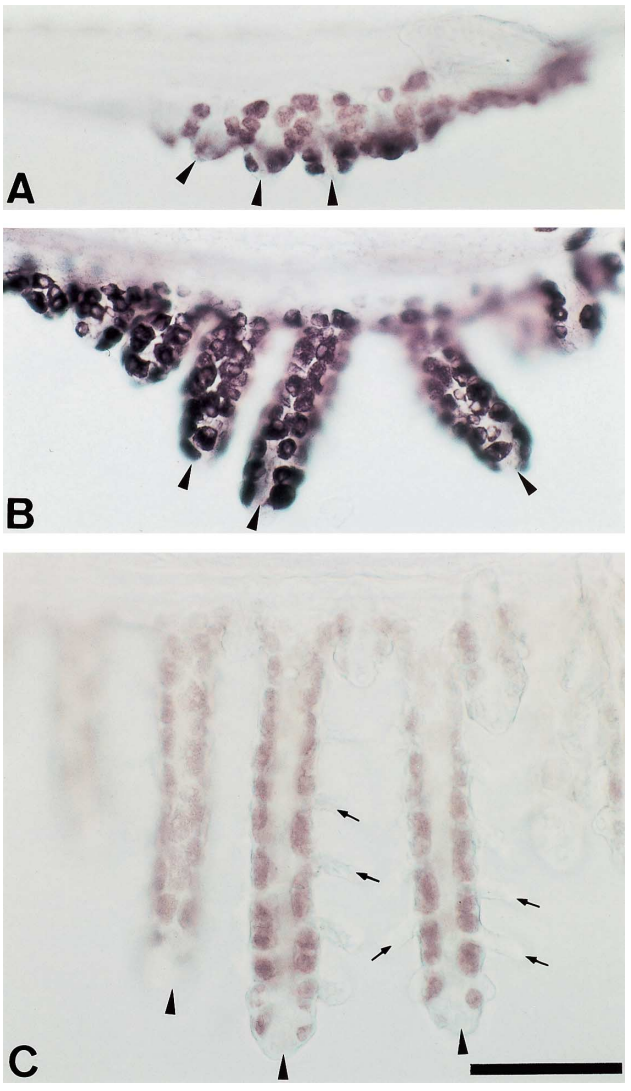

Fig. 3. Branchial chloride cells in the first gill arch of the right side of flounder larvae at day 8 (**A**), day 18 (**B**) and day 24 (**C**), detected by whole-mount immunocytochemistry using an antiserum specific for Na^+,K^+-ATPase. The gill lamellae are already differentiated at day 18 (**B**), although they are not visible because of being out of focus. Arrowheads and arrows indicate gill filaments and lamellae, respectively. Bar: 100 μm.

DISCUSSION

In the present study, the developmental sequence of both cutaneous and branchial chloride cells was clarified in the early life stages of Japanese flounder by the whole-mount immunocytochemistry. As summarized in Table 1, the chloride cell distribution shifts from the body skin to the gills during the early development. Such a spatial shift of chloride cell distribution is closely associated with metamorphosis.

Larvae at days 0 and 4 possessed only cutaneous chloride cells, which seem to be the only functional site for ion secretion at these early developmental stages. In premetamorphic larvae at days 8-18, a large number of chloride cells were observed in both the body skin and gills. Although the mean size of cutaneous chloride cells decreased gradually during the development, the cell density increased and reached the highest value at day 18, just before the beginning of metamorphosis. We did not carry out a quantitative analysis of branchial chloride cells, since it was difficult to measure the precise cell density and size in the complicated, three-dimensional structures of the gills. However, the branchial chloride cell number certainly increased during the development. These findings suggest that both cutaneous and branchial chloride cells function cooperatively as the site for ion secretion at these premetamorphic stages. Later on, cutaneous chloride cells disappeared and branchial chloride cell number increased

further during metamorphosis. These observations clearly indicate that the site for ion secretion shifts from cutaneous to branchial chloride cells during flounder metamorphosis.

During the metamorphosis of Japanese flounder, a series of physiological changes occurs: larval types of the digestive system, muscles and erythrocytes change into adult types (Tanaka, 1973; Yamano *et al*., 1991; Miwa and Inui, 1991; Miwa *et al*., 1992). The shift from cutaneous to branchial chloride cells would be categorized as one of such physiological changes during metamorphosis. Besides Japanese flounder, many other marine teleosts also exhibit metamorphosis to varying degrees. The shift from cutaneous to branchial chloride cells observed in Japanese flounder could be expected to occur during metamorphosis in those teleosts.

A large proportion of cutaneous chloride cells in premetamorphic flounder larvae formed multicellular complexes. Similar chloride cell complexes have been reported in the yolk-sac membrane and body skin of tilapia (*Oreochromis mossambicus*) and Japanese eel (*Anguilla japonica*) embryos and larvae reared in seawater (Shiraishi *et al*., 1997; Sasai *et al*., 1998a). Shiraishi *et al*. (1997) have proved that a chloride cell complex in the yolk-sac membrane of seawater-adapted tilapia larvae consists of a main chloride cell and adjacent accessory cells, which interdigitate with each other to form multiple junctions. The complex is considered to be advantageous to Na^+ secretion, since Na^+ secretion may occur down its electrochemical gradient via a paracellular pathway in the complex (Marshall, 1995; McCormick, 1995). Therefore, the occurrence of chloride cell complexes in the body skin of premetamorphic flounder larvae would provide morphological evidence that those cells function as ion-secreting sites in seawater.

It should be noted that branchial chloride cells first appeared on the gill filaments before the differentiation of lamellae at day 8. Since the lamellae are largely involved in gas exchanges by enlarging the branchial surface area, the primary function of the gills during the early development seems to be ion regulation rather than gas exchanges.

In metamorphic larvae, although the gills were equipped with filaments and lamellae, chloride cells were distributed only in the filaments but not in the lamellae. Two distinct types of chloride cells have been reported in the gill filaments and lamellae of chum salmon (*Oncorhynchus keta*) fry and adults and Japanese eel adults (Uchida *et al*., 1996, 1997; Sasai *et al*., 1998b). In the gills of these species adapted to fresh water, chloride cells were found in both filaments and lamellae. After transfer to seawater, the number of lamellar chloride cells decreased and filament chloride cells were activated. These morphological observations suggest that filament and lamellar chloride cells are involved in seawater and freshwater adaptation, respectively. Therefore, filament chloride cells of flounder larvae may be important in seawater adaptation, most probably acting as the site for salt secretion in hyperosmotic environments. In nature, flounder larvae migrate from offshore areas to estuaries during metamorphosis, when the low-salinity tolerance develops to some extent (Hiroi *et al*., 1997). Observations on branchial chloride cell alteration during the adaptation to hypoosmotic environments would be of considerable interest to explore diverse functions of chloride cells in iono- and osmoregulation.

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